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RESEARCH LABORATORY

# QUARTERLY ENGINEERING PROGRESS REPORT

## STUDY OF BASIC BIO-ELECTROCHEMISTRY

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# ILLUSTRATIONS

## SUMMARY

Work on this program is presently directed to (1) survey of a spectrum of biological systems for selection of a few which are most suitable for the study of bio-electrochemistry and (2) selection of appropriate chemical and electrochemical techniques for carrying out such a study. Preliminary results as obtained to date are generally in accord with those reported by other investigators and have led to the following pertinent observations:

1. All biological oxidation-reduction systems examined were able to produce current under proper conditions.
2. In all cases, performance was improved by an electron transfer agent such as ferricyanide or methylene blue.
3. No induction period for the generation of bio-electrochemistry has been found with any system. The inference is that the source of electrochemical activity is not a long lived intermediate.
4. Only partial correlation is found between electrochemical activity and oxygen uptake by biological oxidation-reduction systems.

Evidence is presently insufficient to provide a clear interpretation of these observations.

# SECTION 1

# INTRODUCTION

This program is concerned with a basic investigation of the biological, chemical, and electrochemical principles involved in the generation of electrical energy by biological systems. It is presently planned to carry out the program in three phases. The first involves the survey of a spectrum of biological systems for the purpose of selecting a few which are most suitable for detailed study. The most promising experimental techniques for the investigation will also be evaluated during this first phase. The second phase will be devoted to a detailed study of the selected systems for the purpose of defining the processes which occur at the bioelectrodes. The third phase will be concerned with the somewhat more practical aspects of the subject such as the optimum physical configuration of bio-electrodes, theoretical efficiencies, and the life and viability of micro-organisms under operating conditions in a bioelectrochemical cell.

The program is presently in the first phase. Since microbiological processes are normally carried out through the medium of various enzymes, the survey has, to date, been restricted to oxidation-reduction enzyme systems. Among those studied are the cell mitochondria, amino acid oxidase, glucose oxidase and some dehydrogenases. Experimental techniques used so far have been restricted to relatively simple

potentiostatic measurements in which current delivered by the biological system is determined as a function of time and at constant electrode potential. Preliminary work has been done on setting up means for carrying out chronopotentiometric studies which should provide a much greater insight into the nature of the electrode processes.

Results obtained to date are generally in accord with those reported by other investigators. They are too incomplete for any definitive conclusions, but are presented in detail in the following sections.

# SECTION 2

# MATERIALS AND METHODS

## 2.1 MATERIALS

Streptococcus faecalis (8043), Proteus vulgaris (13315), Bacillus subtilis (6051) and Endomyces decipiens (Reese)(11647) were obtained from the American Type Culture Collection. D-amino acid oxidase, a crude preparation from hog kidney, and catalase, a purified powder from beef liver, were obtained from Sigma Chemical Co. Glucose oxidase (about 50 percent pure and of fungal origin), alcohol dehydrogenase from yeast (2X crystallized), diaphorase prepared from Clostridum Kluveri, and lactic dehydrogenase from rabbit muscle (2X crystallized) were obtained from Mann Research Laboratories. Flavin adenine denucleotide (FAD), diphosphopyridine nucleotide (DPN) and jack beans were products of CalBioChem. The D-isomers of tryptophan, tyrosine, methionine, alanine, serine, valine, (allo)-isoleucine, phenylalanine and leucine were obtained from Sigma Chemical Co. D-histidine and the dyes methylene blue and 2,6-dichlorophenolindophenol were obtained from K&K Laboratories. The Folin-Ciocalteau Phenol Reagent was obtained from Uni-tech and the 2,4-dinitrophenylhydrazine from Eastment Chemicals. All other chemicals used in the biological studies were reagent grade.

## 2.2 METHODS

### a. ISOLATION OF MITOCHONDRIA AND ASSAY OF RESPIRATORY ACTIVITY

The mitochondria from rat liver were isolated in 0.25M sucrose containing 1 percent serum albumin. The livers were removed from the rats and immediately cooled to 0-4°C in ice cold sucrose solution. Subsequent steps were carried out at 0-4°C. The livers were blotted, weighed, cut into small pieces and placed in the sucrose solution (9 ml for each gram of tissue). The tissue was homogenized for one minute, rechilled and homogenized for an additional minute. The homogenate was centrifuged at 700 x g for 10 minutes. The supernate from the first centrifugation was saved and centrifuged at 5000 x g for 10 minutes. The pellets from this centrifugation were redispersed by homogenization and centrifuged at 24,000 x g for 10 minutes. The sediment was redispersed and centrifuged twice more at 24,000 x g. The final pellets were suspended in 0.25M sucrose containing 1 percent serum albumin and were employed as such in the studies.

The mitochondria from the white buds of cauliflower were isolated in 0.5M sucrose containing 0.001M sodium versenate and 1 percent serum albumin by the method of F. L. Crane[1] with several modifications. The cauliflower was ground in the Waring Blendor for 20 seconds at full speed. The resulting solution was centrifuged at 2000 x g for five minutes the precipitate discarded and the supernate centrifuged at 5000 x g for ten minutes. The mitochondria pellet was resuspended in the sucrose solution and used in the studies.

The respiratory activity of the mitochondria isolated from rat liver, was determined in the Warburg apparatus. The main compartment contained 1.0 ml of 0.1 M disodium succinate in buffer, 1.0 ml of 0.5 M phosphate buffer pH 7.4 and 1.0 ml of mitochondrial solution. The center well contained 0.2 ml of 2N NaOH plus a 1 $cm^2$ filter paper wick. The temperature of the bath was 38°C and the gas phase was air. A ten minute equilibration period was employed from addition of mitochondria until the first manometer reading.

Oxygen consumption by the mitochondria isolated from cauliflower buds was also measured in the Warburg apparatus. The main compartment of the reaction vessel contained 20 mM succinate, 5 mM $MgCl_2$, 1 mM EDTA and 5 mM phosphate buffer at pH 7.1 in a total volume of 3 ml. The mitochondria preparation (1 ml) was added five minutes before the first manometer reading was made. The center well contained 0.2 ml of 2N NaOH with a 1 $cm^2$ filter paper wick. The bath temperature was 25°C with a gas phase of air.

When mitochondria were isolated from either rat liver or cauliflower buds a quick qualitative test was used to determine the oxidative activity of the preparations. Eight drops of a 30mg/0.1 ml solution of Janus green was added to the mitochondrial suspension and the mixture centrifuged. The pellet, which resulted contained a mixture of mitochondria and Janus green dye, was then incubated at 37°C. Mitochondria of high activity caused the color of the mitochondrial pellet to change from blue to red in less than 10 minutes.

b. ENZYME ISOLATION AND ASSAY

The isolation of D-amino acid oxidase from frozen hog kidney was carried out according to the method of Massey, Palmer and Bennet.[2] The procedure is a combination of heat and salt precipitation and was completed to the next to last step of Stage 3 of their purification scheme. The resulting solution had an activity of over 300 $\mu$l $O_2$/30 min./mg protein as determined by oxygen consumption in the Warburg apparatus. Assays were carried out under the following conditions. The main compartment contained 0.1 ml of $10^{-4}$ M FAD, 1 ml enzyme solution and 1.7 ml of 0.1M pyrophosphate buffer, pH 8.3, containing 0.1 mg/2 ml of catalase, the center well had 0.2 ml of 2N NaOH plus a 1 $cm^2$ filter paper wick, the side arm contained 0.2 ml of 2.5 percent D-alanine. The gas phase was air and the temperature was maintained at 37°C. The manometers were shaken for two minutes, the contents of the side arm mixed with the contents of

the main compartment, the shaking resumed and after five minutes the first manometer reading was made.

The reaction of D-amino acid oxidase was also assayed by determination of products and substrates. Quantitative estimation of free keto acids involved the use of 2,4-dinitrophenylhydrazine. To one ml of solution containing keto acid was added 6 ml of 0.025 percent 2,4-dinitrophenylhydrazine and 2 ml of 40 percent KOH. After 10 minutes at room temperature the color intensity was determined at 450 $\mu$. The control contained 0.1 M pyrophosphate buffer pH 8.3. Quantitative paper chromatographic determination of the free amino acids was carried out by the method of Kay, Harris and Entenman.[3]

Urease was isolated from jack beans by the method of K. Hanabusa.[4] The isolation was carried as far as crude crystals. Urease activity was determined in the Warburg apparatus by the measurement of $CO_2$ produced. The main compartment contained 3 ml of 3N sodium acetate buffer (pH 5.0) containing 100 mg urea. Urease solution (0.2 ml) was added and the assay run at 38°C with air as the gas phase. Manometer readings were made at one minute intervals.

Glucose oxidase activity was determined with the Warburg apparatus. The main compartment contained 2.8 ml of 0.2 M potassium phosphate buffer pH 5.6 containing 0.018 mg of Glucose Oxidase. The side arm had 0.2 ml of buffer containing 4 mg of glucose. The center well had 0.2 ml of 2N NaOH with a 1 $cm^2$ filter paper wick. The determination was run at 25° with air as the gas phase. The flasks were equilibrated for two minutes and the contents of the main compartment and the side arm mixed. The initial manometer reading was made after two minutes additional shaking.

The activity of yeast alcohol dehydrogenase was measured by the rate of reduction of DPN as indicated by the increase in absorbancy at 340 $\mu$ . In the spectrophotometer cuvette 2.7 ml of 0.01 M pyrophosphate buffer at pH 8.5, 0.1 ml of 3 M ethanol and 0.1 ml of 0.0015 M DPN were combined and mixed. At zero time 0.1 ml of enzyme solution (1 mg/ml) was

added to the cuvettes. Optical density determinations were made at 15 second intervals.

The activity of lactic acid dehydrogenase was also measured by the rate of increase in absorbancy at 340 $\mu$ . In the spectrophotometer cuvette is placed 2.7 ml of 0.003 M phosphate buffer pH 7.4, 0.1 ml of 0.01 M sodium lactate and 0.1 ml of 0.002 M DPN. At zero time 0.1 ml of enzyme solution (0.5 - 1.0 $\mu$g) is added. Optical density readings were made at 15 second intervals.

Diaphorase activity was determined by following the reduction of 2,6-dichlorophenolindophenol at 600 $\mu$ . In this determination 0.1 ml of 0.2 M Tris buffer at pH 7.5, 0.1 ml of 0.006 M DPNH, 0.1 ml of 0.0012 M 2,6-dichlorophenol indophenol and 2.5 ml of distilled water were combined in the spectrophotometer cuvette. At zero time 0.1 ml of enzyme solution was added to the cuvette. Optical density determinations were made at 30 second intervals.

The method of Lowry et al[5] was employed for all protein determinations.

c. GROWTH AND STORAGE OF MICRO-ORGANISMS

Proteus vulgaris and Bacillus subtilis were grown in a medium of 1 percent tryptone, 0.5 percent yeast extract and 0.5 percent sodium chloride. To ensure against loss of bacterial strains, bacteria were grown, lyophilized, sealed in vacuo and stored in the cold. The bacteria were grown in 125 ml of media in 500 ml elenmeyer flasks until the optical density of the solution at a wavelength of 600 $\mu$ reached 0.6. The bacteria solution was centrifuged at 10,062 x g for five minutes. The precipitate from six 500 ml flasks were resuspended in 5 ml of a solution containing 0.5 percent ascorbic acid, 2.0 percent dextrin, 0.5 percent thiourea and 0.5 percent $NH_4Cl$ with the pH adjusted to 7.0. The bacteria suspension was put into flasks, shell frozen in dry ice and butanol, placed on a vacuum manifold with the bottom of the tubes in

frozen carbon tetrachloride (-26°C) and left until the contents were dry. The tubes were sealed by flame while still on the vacuum manifold.

When a pellicle was desired the Bacillus subtilis was grown without shaking. Two tests were used to characterize Proteus vulgaris. Urease production by Proteus vulgaris was tested using Christensen's medium and hydrogen sulfide production was demonstrated using lead acetate agar.

d. POLARIZATION MEASUREMENT OF BIO-ELECTRODE

Polarization measurements of the bio-electrode were conducted with the apparatus schematically illustrated in Figure 1. The potential between the test electrode $e_1$ and the reference $e_2$ (Saturated calomel electrode, SCE) was maintained at +0.200 volt by changing the current through the electrochemical cell. The polarizing current required to maintain this potential was obtained by adjusting the variable resistance R manually. The value of 0.200 volts was selected for the measurements since at this potential the current output of the bio-electrode seemed to show the greatest variation between individual systems.

Detailed configuration of the polarization cells used in the investigation are shown in Figures 2 and 3. Temperature was maintained by circulating constant-temperature water (at 38°C.) through the outer jacket.

The electrode material employed in all tests was a platinized platinum (platinum black) electrode. The composition of the anolyte solutions for the D-amino acid oxidase system was 0.1 M sodium pyrophosphate buffer (pH 8.3), 2 to 3 mg/ml substrate and either 0.2 mg/ml of purified D-amino acid oxidase or 2 mg/ml of the commercially available D-amino acid oxidase. The test solution was degassed by boiling at ambient temperature in vacuo and bubbling nitrogen gas through it prior to addition of the enzyme. In the glucose oxidase experiments the reaction mixture was composed of 0.1 sodium phosphate buffer pH 5.7,

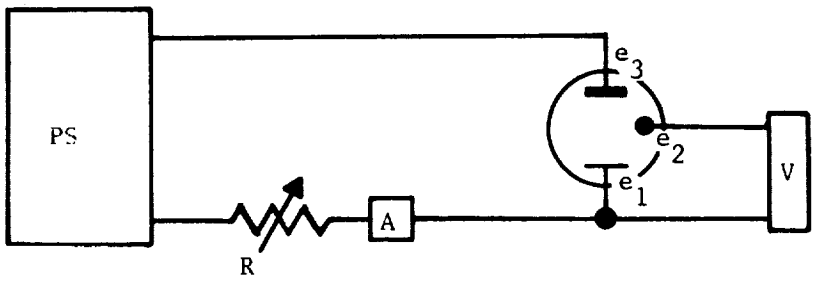


FIGURE 1. SCHEMATIC DIAGRAM OF APPARATUS USED FOR POLARIZATION MEASUREMENT OF BIO-ELECTRODE

PS = HEWLETT-PACKARD 711A D.C. POWER SUPPLY, A = SIMPSON 269 MICROAMMETER VOM, V = HEWLETT-PACKARD 412A VACUUM TUBE VOLTMETER, R = INDUSTRIAL INSTRUMENT DECADE RESISTANCE BOX, $e_3$ = AUXILIARY ELECTRODE, $e_2$ = REFERENCE ELECTRODE (SCE), $e_1$ = TEST ELECTRODE (PLATINIZED PLATINUM DISC)

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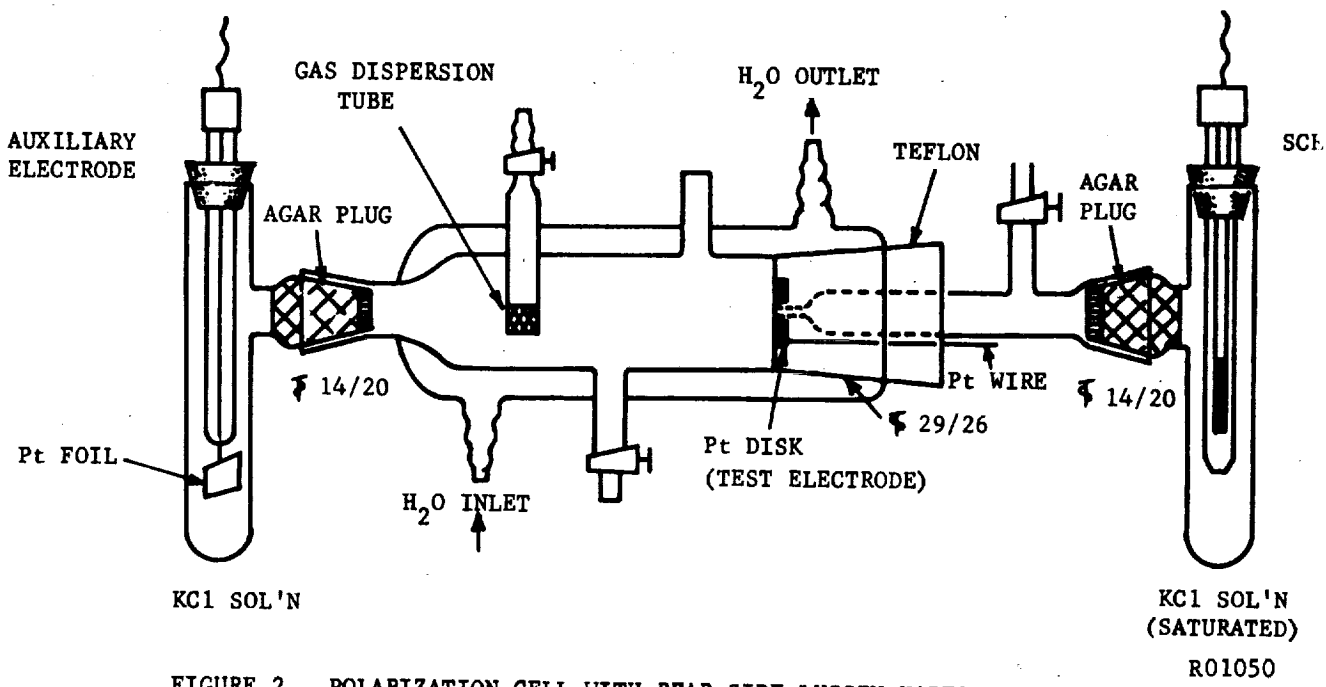


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FIGURE 2. POLARIZATION CELL WITH REAR SIDE LUGGIN-HABER CAPILLARY

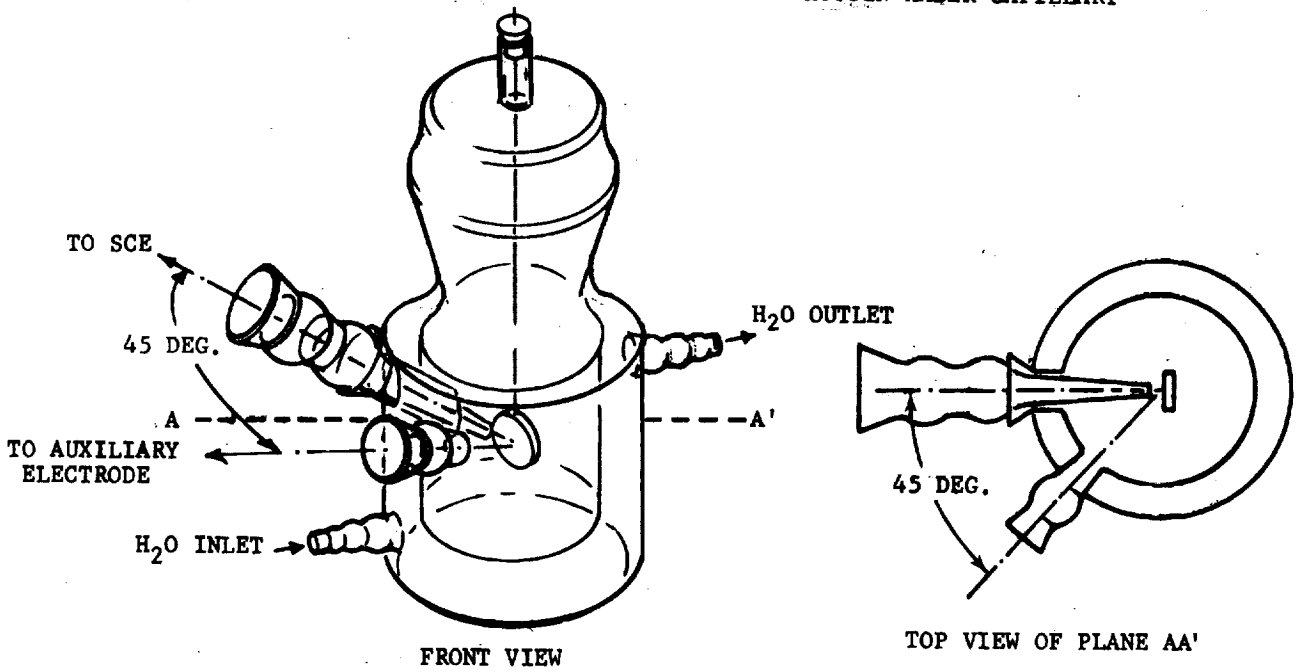


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FIGURE 3. POLARIZATION CELL WITH FRONT SIDE LUGGIN-HABER CAPILLARY

0.1 M glucose, 0.01 M methylene blue and 0.24 mg/ml glucose oxidase. This solution was also degassed before the addition of enzyme. When mitochondria were employed, the cell contained 150 mM sodium phosphate buffer pH 7.4, 30 mM disodium succinate and a 1 to 3 dilution of the mitochondria preparation.

The current density obtainable from an electrochemical cell normally is dependent upon stirring when the latter is below a certain limiting rate. It is customary to make all observations under conditions of stirring in excess of this limiting rate. In the present work, however, it was found that some dependence of cell current on stirring still remained at the highest rates obtainable. In order to minimize the possibility of extraneous effects from this situation, care was taken to maintain, insofar as possible, identical stirring conditions in all experiments.

e. STUDY OF BIO-ELECTRODE PROCESSES BY CHRONOPOTENTIOMETRY

In order to seek an understanding of the chemical and electrochemical processes taking place in the bio-electrode, chronopotentiometric studies were initiated during this quarter. The successful application of this experimental technique to the study of various electrode reactions has been amply demonstrated.[6-9]

The principle of chronopotentiometry is based upon constant-current, diffusion-controlled electrolysis in unstirred solutions. A thorough theoretical discussion of the significance of the chronopotentiograms obtained under these conditions has been given by Delahay and coworkers,[6,10] and by Reinmuth.[11,12] According to these investigators, an analysis of the transition time ($\tau$) and the potential-time curve should provide valuable information about the kinetics of chemical and electrochemical reactions.

Two general classes of reactions exist. First, in cases in which kinetic complication other than simple diffusion follows the charge transfer reaction, the transition time is unaffected by the complication.

For this situation the detection of the kinetic complication rests upon the careful scrutiny of the potential-time curves. For ease in comparison, Reinmuth[11] has summarized the diagnostic criteria and the potential-time relations for various kinetic schemes.

The second class of reactions are those in which some complication other than pure diffusion precedes electron transfer; here the kinetic scheme may be distinguished by the inconstancy of $i\tau^{\frac{1}{2}}$ with varying current (i). Distinction between the various reaction schemes can be made on the basis of two experimental plots: $i\tau^{\frac{1}{2}}$ vs i at constant C (concentration) and $\tau^{\frac{1}{2}}$ vs C at constant i. The diagnostic criteria to be applied to these plots have been developed by Reinmuth.[12]

Although in both classes of reactions the diagnostic criteria and the potential-time relations were developed for chronopotentiometric reduction mechanisms, these can be applied with slight modifications to oxidation processes. If it is found in the present studies that the oxidation process at the bio-electrode falls within one of the derived diagnostic critera, effort will then be made to determine the reaction parameters.

Experimental studies were performed with the instrument schematically represented in Figure 4. The cell is connected to a voltage regulated power supply PS (Hewlett-Packard 711A) through a large series resistor $R_1$. This series resistor insures constant current to the cell. The potential of the test electrode $e_1$ is measured against a reference electrode $e_2$ (SCE) by means of recorder R (Moseley Autograf X-Y Recorder, Model 2DR-2). A preamplifier P having an input impedance of more than 100 megohms is used in the circuit between the test electrode and the reference electrode to minimize the flow of current between these electrodes. With resistor $R_2$ in the circuit, current to be used for the electrolysis can be preselected. Resistor $R_2$ is adjusted so that when DPDT switch is thrown from position $S_1$ to $S_2$ substantially no change in current is observed on the ammeter A (Simpson 269 Microammeter VOM).

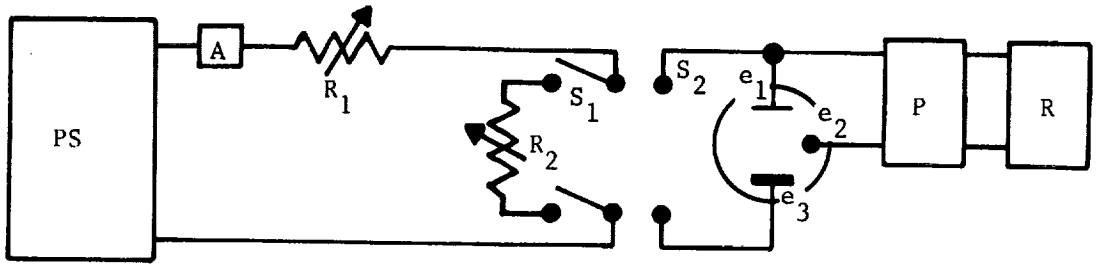


FIGURE 4. SCHEMATIC DIAGRAM OF APPARATUS USED FOR CHRONOPOTENTIOMETRY

PS = HEWLETT-PACKARD 711A D.C. POWER SUPPLY, A = SIMPSON 269 MICROAMMETER VOM, $R_1$ AND $R_2$ = CLAROSTAT VARIABLE RESISTORS, P = PREAMPLIFIER, R = MOSELEY AUTOGRAF X-Y RECORDER, MODEL 2DR-2, $e_1$ = TEST ELECTRODE, $e_2$ = REFERENCE ELECTRODE (SCE), $e_3$ = AUXILIARY ELECTRODE, $S_1$ AND $S_2$ = POSITIONS OF DPDT SWITCH

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Prior to electrolysis, current is passed through $R_1$ and $R_2$ until these resistors attain thermal equilibrium. Then the pen drive on the recorder is started and at some arbitrary time the electrolyzing current is passed through the cell (via electrode $e_1$ and $e_3$) by throwing the DPDT switch from position $S_1$ to $S_2$ and the potential-time curve is recorded.

Electrolysis was conducted under anaerobic conditions. The cell was thermostated by circulating constant-temperature water through the outer jacket.

# SECTION 3

# RESULTS

## 3.1 MITOCHONDRIAL PREPARATIONS

As previously described, preparations of mitochondria were isolated from rat liver and cauliflower. On the basis of oxygen consumption, as determined in the Warburg apparatus, it was found that both cauliflower and liver mitochondria could be stored in the frozen state for 72 hours with a decrease in activity not exceeding 50 percent. The whole cauliflower can be stored for at least 24 hours before isolation of mitochondria without decrease in respiration rate. Rat livers cannot be excised and stored without the subsequently prepared mitochondria losing all of their respiratory activity. Bovine serum albumin at 1 percent concentration is beneficial to both types of mitochondria in improving storage characteristics and maintenance of activity during incubation. The liver mitochondria with 1 percent serum albumin evidenced an 80 percent decrease in activity during a 4 and 1/2 hour incubation. Without serum albumin the activity showed a decrease in excess of 93 percent. These results demonstrated that the mitochondria could be effectively employed for long term studies in the electrochemical cell.

Mitochondria were tested for their ability to produce a current in the electrochemical cell. Rat mitochondria supplied with succinate as an energy source failed to develop a current under the conditions

outlined in Section 2.2 a. Low respiratory activity of the mitochondrial preparation would not appear to be responsible for the lack of current since the preparation was found to have a respiratory rate of 30 $\mu$ liters $O_2$/min/ml by manometric assay. However, the addition of ferricyanide (0.003M) to the mitochondrial anolyte solution as described in Sections 2.2 a. resulted in a current of 85 $\mu$amp/cm$^2$, which increased to 160 $\mu$amp/cm$^2$when the ferricyanide concentration was increased to (0.006M) and to 225 $\mu$amp/cm$^2$ with (0.009M) ferricyanide. These currents were constant over periods of observation up to 25 min. These results are depicted in Figure 5, where the true current-time curve is shown as a series of discontinuous steps, due to the fact that with the manual potentiostat used it was impossible to follow the rapid rise in current upon adding the ferricyanide.

The conclusion drawn is that intact mitochondria cannot support a current under the conditions of the experiment in the absence of an intermediate electron acceptor such as ferricyanide.

## 3.2 D-AMINO ACID OXIDASE

Commercially available D-amino acid oxidase was employed in the initial studies on this enzyme. Manometric assay determined the activity to be 0.5 $\mu$ liters $O_2$/min/mg protein. As shown in Figure 6, the enzyme activity based on respiratory action ($O_2$ uptake) decreased 37 percent in the first 15 minutes, but only an additional 10 percent in the next five hours. This result was promising as it indicated that long term studies in the electrochemical cell would be possible. It was found that for adequate activity flavin adenine dinucleotide had to be added to the enzyme preparation.

In spite of the significant activity shown in the manometric assay, the enzyme (1 mg/ml) with D-alanine as a substrate gave no measurable current in the electrochemical cell. Methylene blue was tested for its ability to act as an intermediate between the enzyme and the electrode and

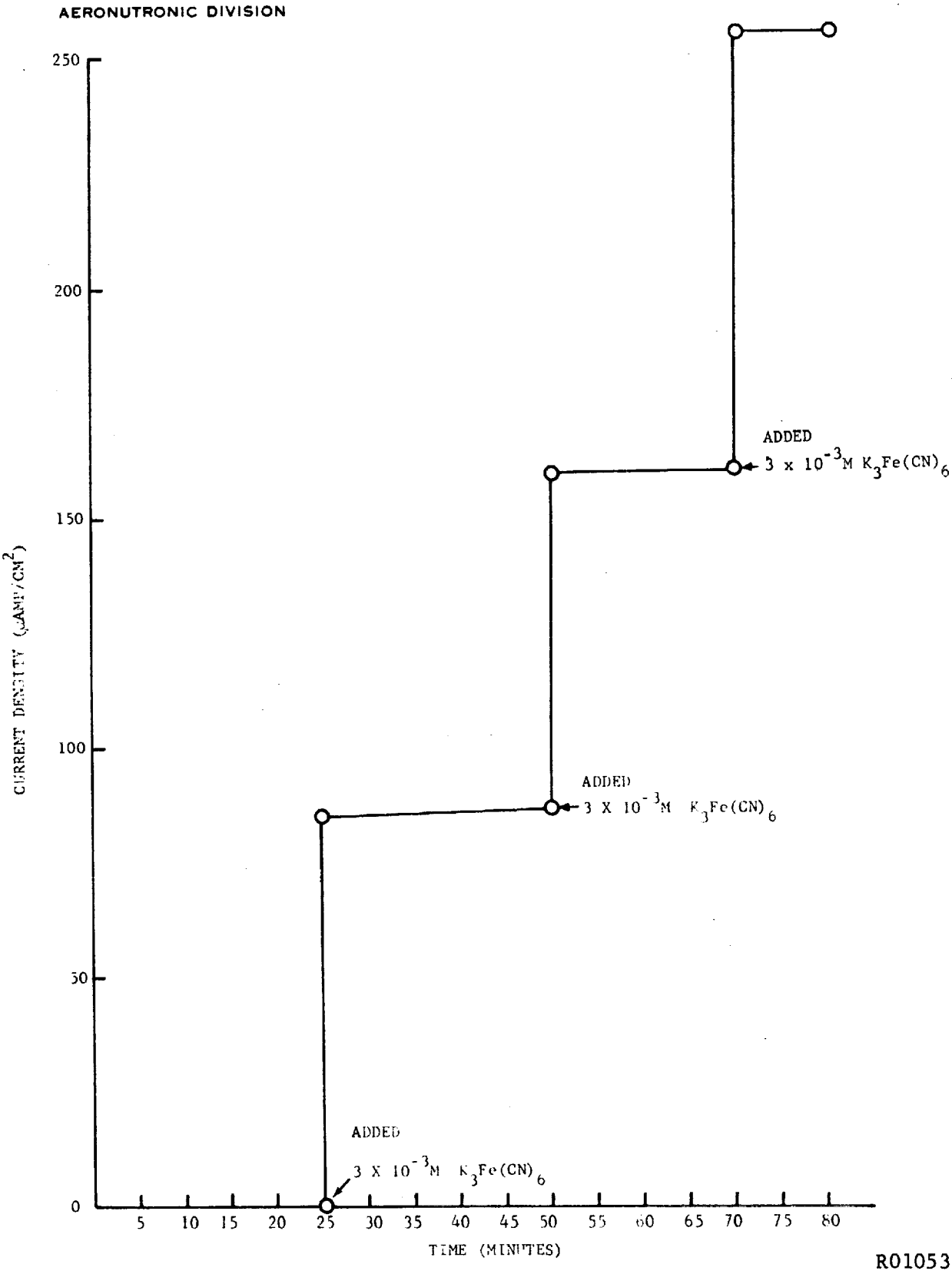


FIGURE 5. EFFECT OF $K_3Fe(CN)_6$ UPON ELECTROCHEMICAL ACTIVITY OF MITOCHRONIDA

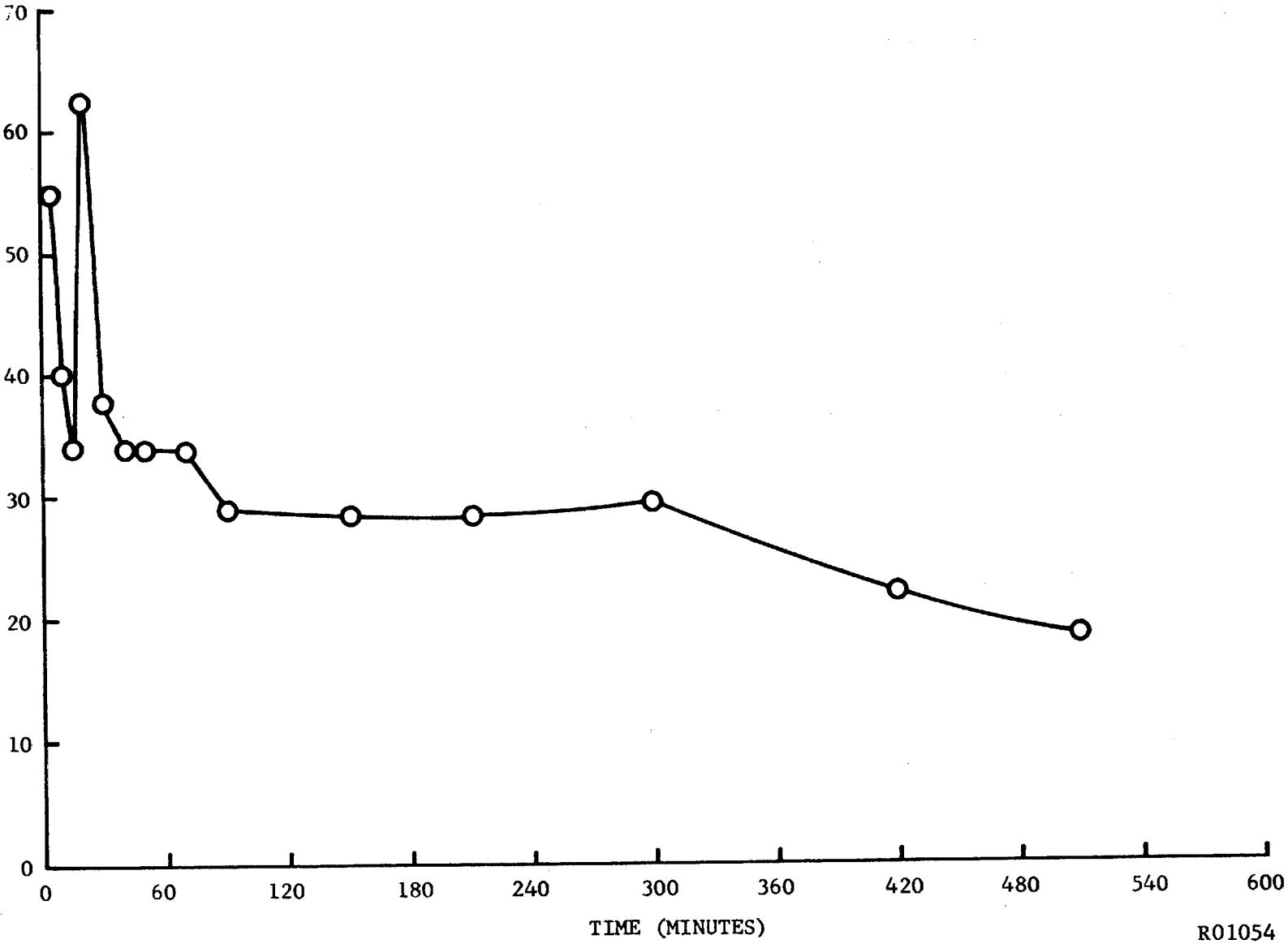


FIGURE 6. DECAY IN ACTIVITY ($O_2$ UPTAKE) OF COMMERCIAL PREPARATION OF D-AMINO ACID OXIDASE. SUBSTRATE - ALANINE

was found to yield no improvement. It was suspected that these results might be due to lack of purity of the crude commercial enzyme and that the experiments should be repeated using enzyme freshly prepared from frozen hog kidneys.

Three D-amino acid oxidase preparations were accordingly isolated from frozen hog kidneys according to the procedure of Massey et al[2] as previously discussed. These had respiratory activities of 11.1, 11.0 and 10.7 $\mu$ liters $O_2$/min/mg protein. This enzyme preparation, at a concentration of 0.2 mg protein/ml and with alanine as substrate, showed little improvement in producing a current (3-5 $\mu$ amp/cm$^2$) compared with the crude enzyme at a concentration of 1 mg/ml. Ferricyanide was tested with the Aeronutronic preparation to determine if it could function more effectively than methylene blue as an intermediate between the enzyme and the electrode. When ferricyanide (0.003M) was added to the enzyme-substrate system, the current which could be drawn increased from 3 $\mu$ amp/cm$^2$ to 200 $\mu$ amp/cm$^2$ (Figure 7). This would indicate that ferricyanide can act more effectively as an electron acceptor for the enzyme than can the platinum electrode.

Studies have been carried out with D-amino acid oxidase to determine the substrate specificity with respect to the electrode reaction, and compare this with specificity observed in respiratory activity. In this work an intermediate electron acceptor (i.e., ferricyanide or dye) was not present. The substrates tested were the D-isomers of tryptophan, histidine, serine, alanine, valine, methionine and leucine.

The results shown in Figure 8 indicate that marked differences in electrode activity occur with different substrates. Tryptophan is obviously most effective. The results with tyrosine do not take into account its limited solubility. With oxygen as an acceptor the substrate specificity of D-amino acid oxidase was determined with tryptophan, serine and alanine and the results are shown in Figure 9. Serine and tryptophan were chosen for comparison because they represented the two

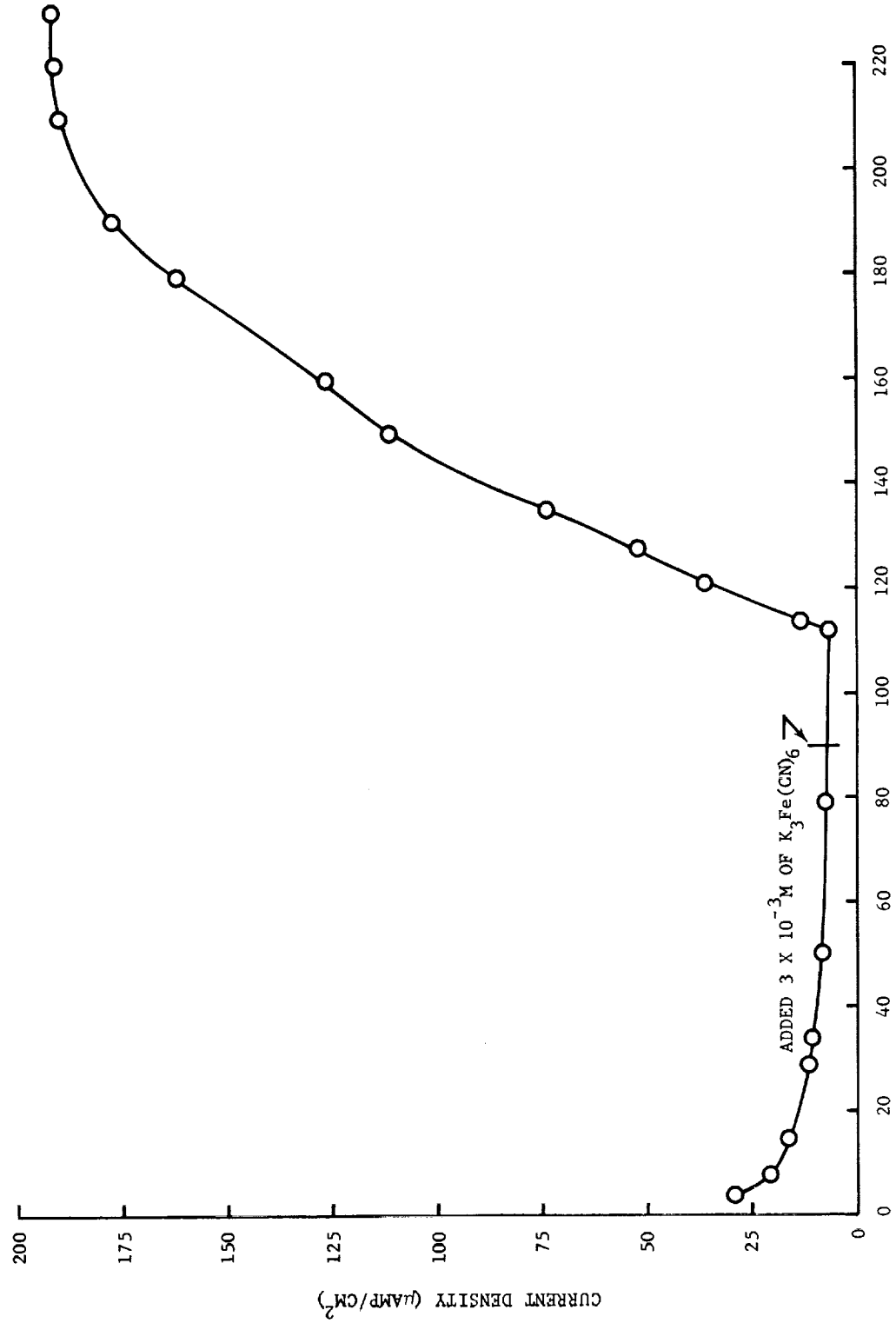


FIGURE 7. IMPROVEMENT IN ELECTROCHEMICAL ACTIVITY OF D-AMINO ACID OXIDASE (AERONUTRONIC PREP.) UPON ADDITION OF $K_3Fe(CN)_6$ SUBSTRATE - ALANINE

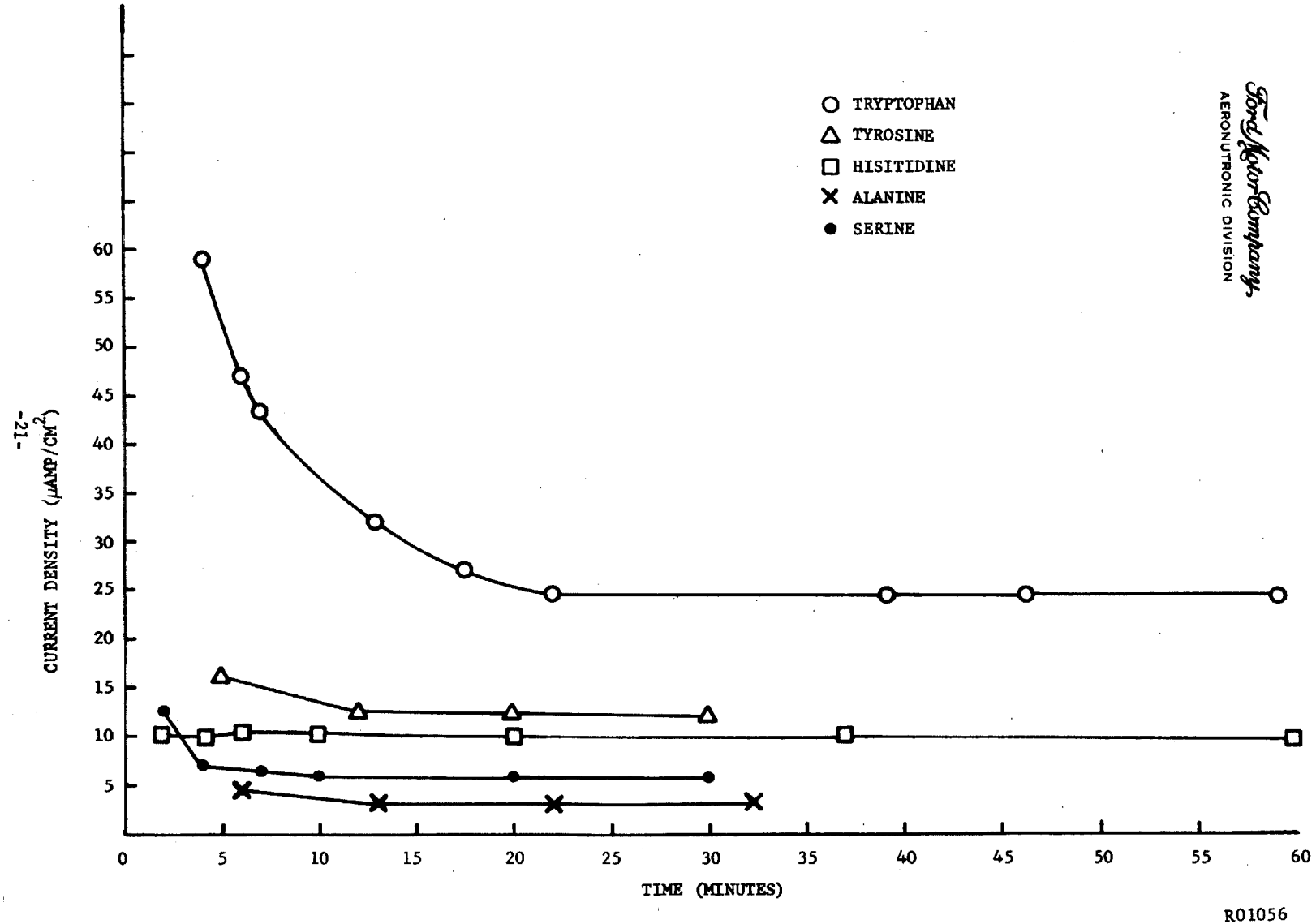


FIGURE 8. ELECTROCHEMICAL ACTIVITY OF D-AMINO ACID OXIDASE WITH DIFFERENT SUBSTRATES

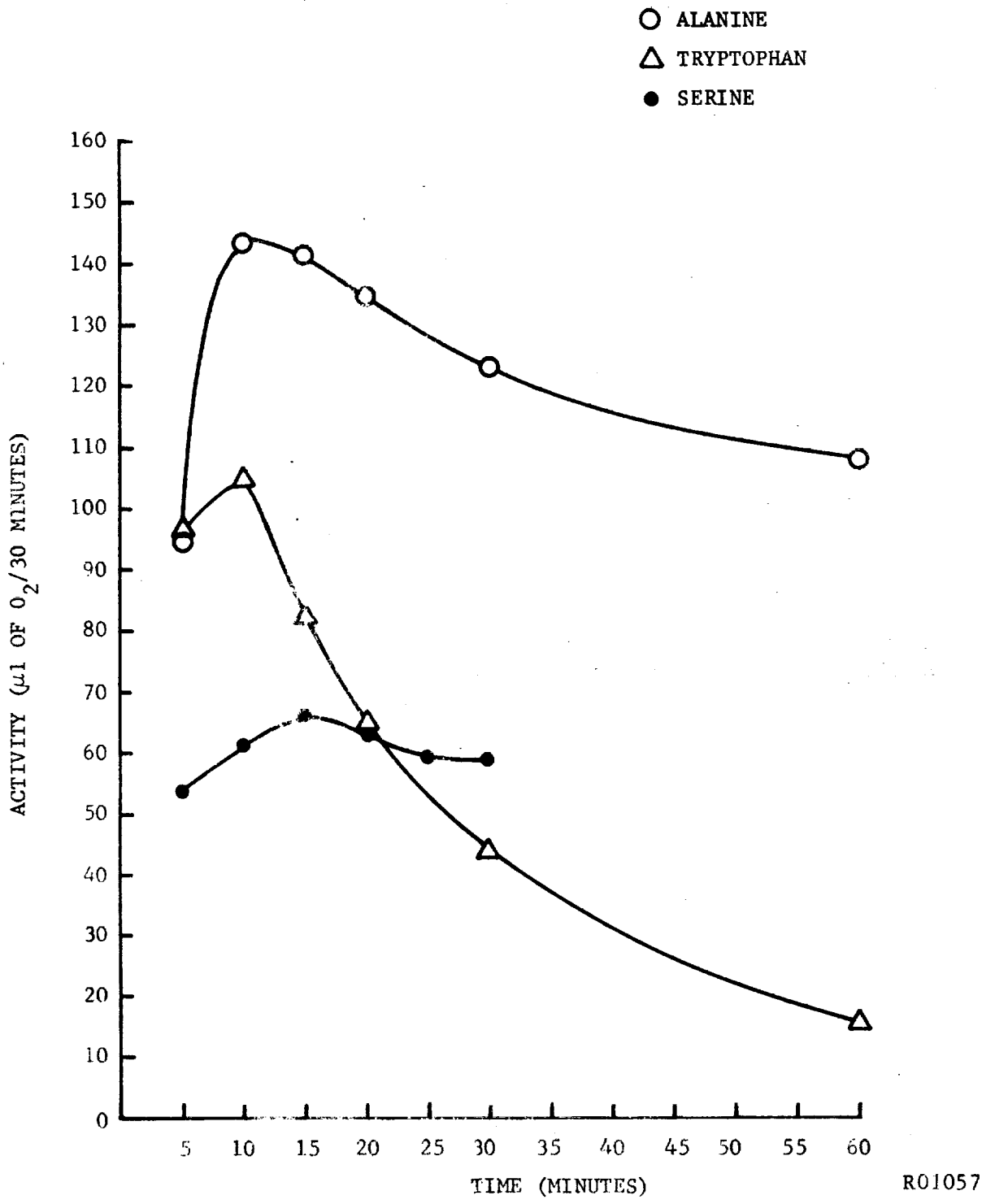


FIGURE 9. OXYGEN UPTAKE OF D-AMINO ACID OXIDASE WITH DIFFERENT SUBSTRATES

extremes of activity at the electrode. Alanine was included because it is the amino acid generally employed for assay of D-amino acid oxidase.

Comparison of the curves of Figure 8 with those of Figure 9 shows only partial correlation between the rate of reaction of a given substrate-enzyme system with oxygen as an electron acceptor and with the platinum electrode of the electrochemical cell. Serine is a relatively poor substrate for productionof current, and is also a poor substrate with oxygen. Tryptophan gives, with oxygen, a lower reaction rate than alanine, a higher initial rate and a lower final rate than serine, but is superior to both serine and alanine for production of electrical current. Comparison of the two curves for tryptophan shows some similarity in shape and in a rapid decrease in activity with time.

The examination of additional substrates with D-amino acid oxidase will be required before definite conclusions can be reached as to the correlation between the activity of the enzyme-substrate system in terms of oxygen absorption, and in terms of the production of electric current. It is possible that analytical studies of the reaction products will provide some rationale for the observed effects.

Subsequent further comparison between the commercial preparation of D-amino acid oxidase, and that prepared at this laboratory, using tryptophane as the substrate, showed that when the concentration of the two enzymes were adjusted to produce the same activity (determined by $O_2$ uptake) they would produce comparable electrical current. Some differences were apparent in the manner in which current developed. With the Aeronutronic preparation, a large initial current developed rapidly and decayed within 20 minutes to a steady level. With the commercial enzyme preparation, the current developed more slowly, ultimately arriving, however, to the same final value. This behavior is shown on Figure 10.

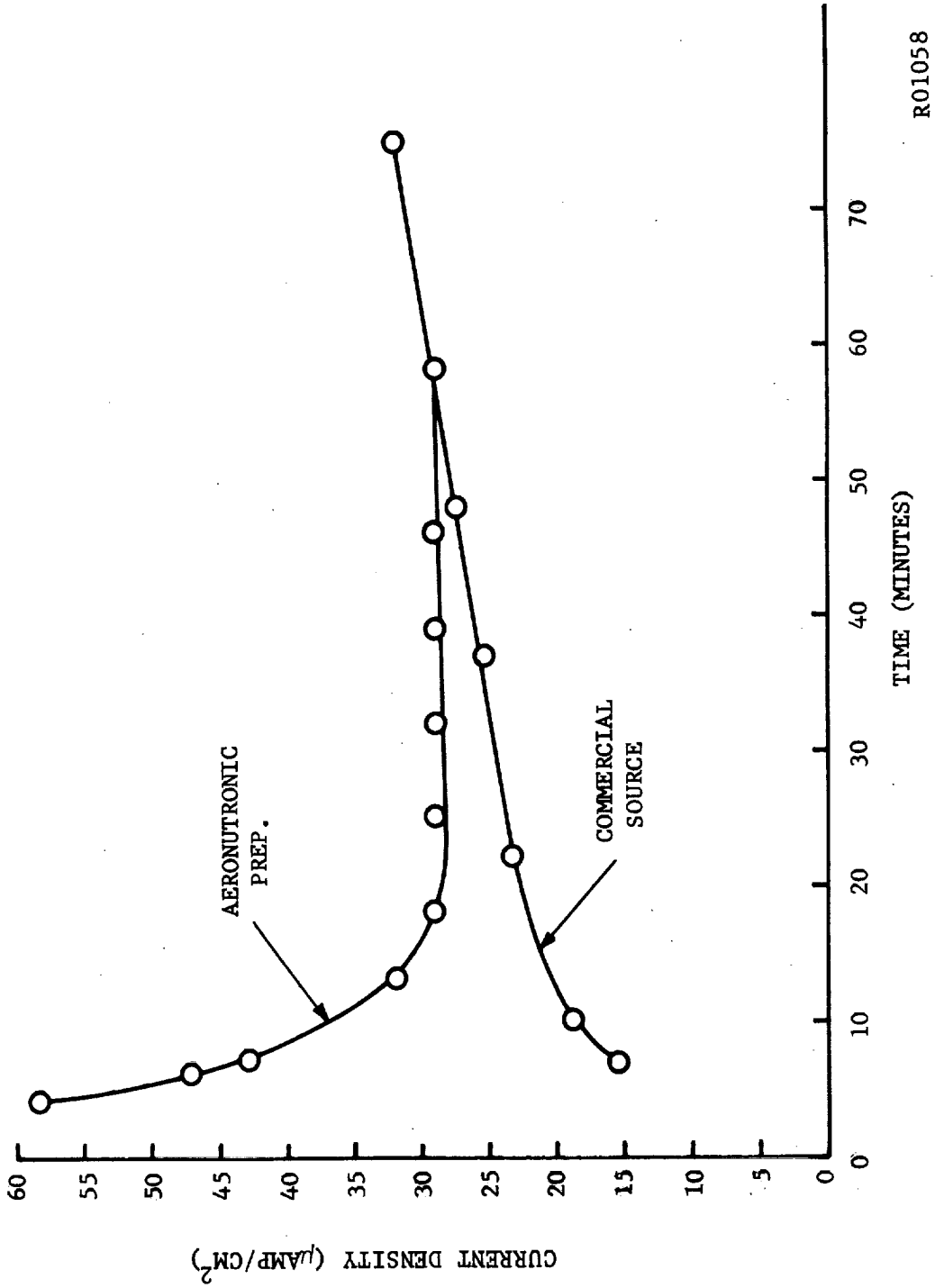


FIGURE 10. ELECTROCHEMICAL ACTIVITY OF D-AMINO ACID OXIDASE FROM TWO SOURCES. SUBSTRATE-TRYPTOPHANE

### 3.3 GLUCOSE OXIDASE

Glucose oxidase (0.4 mg/ml) has been tested in the electrochemical cell with glucose as the substrate. No current was produced in the absence of a charge transfer agent. When methylene blue was introduced, a relatively high current could be drawn, but not for a sustained period. The enzyme reacted rapidly with the methylene blue. This fact was demonstrated by the color change of the reaction mixture from dark blue to a pale green. Along with the color change, there was a simultaneous formation of a precipitate. This precipitate, the leuco form of the dye, suggests the reduced form of the dye was not able to react at a sufficient rate with the electrode and precipitated from solution. The glucose oxidase has not yet been tested with ferricyanide as an oxidation-reduction intermediate.

### 3.4 REACTION BETWEEN THE ENZYME SUBSTRATE SYSTEM AND FERRICYANIDE

The substantial increase in current which has been obtained upon the addition of ferricyanide to the anolyte solution in the electrochemical cell leads to some interest in the nature of the reaction between ferricyanide and biological systems. If it can be assumed that the electrode process

$$Fe(CN)_6^{-4} \rightarrow Fe(CN)_6^{-3} + e \qquad (1)$$

is more rapid than the biological reaction, then the potential of an electrode in a biological system containing ferricyanide is given by the equation:

$$E = E_o + \frac{RT}{n\mathcal{F}} \ln \frac{Fe(CN)_6^{-3}}{Fe(CN)_6^{-4}} \qquad (2)$$

The change in potential with time is therefore a measure of the reaction rate between the ferricyanide and the biological system.

Since in a given situation

$$Fe(CN)_6^{-4} + Fe(CN)_6^{-3} = \text{constant } (S)$$

the above equation can be written

$$E = E_o + \frac{RT}{n\mathcal{F}} \ln \frac{1}{\frac{S}{x} - 1} \tag{3}$$

where

$$x = Fe(CN)_6^{-3}$$

and

$$\frac{dE}{dt} = \frac{RT}{n} \frac{S}{(S-x)x} \frac{dx}{dt} \tag{4}$$

where $\frac{dx}{dt}$ is the rate of conversion of ferricyanide to ferrocyanide. For the cases where this rate is zero order and first order to ferricyanide, and zero order in enzyme concentration:

zero order in $Fe(CN)_6^{-3}$: $\quad -\frac{dx}{dt} = k_o, \qquad x = S - k_o t$

first order in $Fe(CN)_6^{-3}$: $\quad -\frac{dx}{dt} = k_1 x, \qquad x = Se^{-k_1 t}$

Combination with Equation 4 gives, respectively

$$\frac{dE}{dt} = \frac{-S}{t(S-k_o t)} \cdot \frac{RT}{n\mathcal{F}} \tag{5}$$

$$\frac{dE}{dt} = \frac{-k_1}{1 - e^{-k_1 t}} \cdot \frac{RT}{n\mathcal{F}} \tag{6}$$

If the rate is first order in enzyme concentration one obtains the corresponding expressions:

zero order in $Fe(CN)_6^{+3}$: $\quad -\frac{dx}{dt} = k_o Z, \qquad x = S - Zk_o t$ (7)

first order in $Fe(CN)_6^{+3}$: $\quad -\frac{dx}{dt} = k_1 Zx, \qquad x = Se^{-k_1 Zx}$ (8)

where Z = enzyme concentration.

Combination of 7 and 8 with 4 gives

$$\frac{dE}{dt} = \frac{-S}{t(S-Zk_o t)} \cdot \frac{RT}{nF} \qquad (9)$$

$$\frac{dE}{dt} = \frac{-K_1 Z}{1-e^{-Zk_1 t}} \cdot \frac{RT}{nF} \qquad (10)$$

Equations 5 and 6 show that the slopes of curves of voltage vs time are initially independent of S. As time proceeds, however, the (negative) slope will decrease with increasing S for the zero order cases but not for the first order cases. These equations also show that if the rate is zero order in the enzyme concentration, the slope $\frac{dE}{dt}$ will be independent of Z at all time. Equations 9 and 10 show, however, that if the rate is first order in Z, then the effect of changing Z depends upon the order with respect to $Fe(CN)_6^{-3}$. In case the latter is zero order the slope of the voltage-time curves is initially independent of Z, but at later times the slope becomes dependent on S sooner with increasing Z. If the change of voltage with time is first order in ferricyanide then the slope is normally proportional to Z, but falls more rapidly with increasing Z during the initial period.

Figure 11 shows some data obtained on the amino acid oxidase (Aeronutronic preparation)-alanine system in an attempt to obtain information on the reaction with ferricyanide. Curves of voltage vs time were obtained for two ferricyanide concentration, $3x10^{-3}$ and $6x10^{-3}$M, and two enzyme concentrations, 40 ml and 10 ml of enzyme preparation per liter. Curves 2 and 3 were obtained at the same enzyme concentration but at different ferricyanide concentrations. Curves 1 and 2 were obtained at the same ferricyanide concentration, but at different enzyme concentrations. All other conditions were the same.

Except for the start of the run, where measurements may be uncertain due to extraneous electrode reactions or other transient effects,

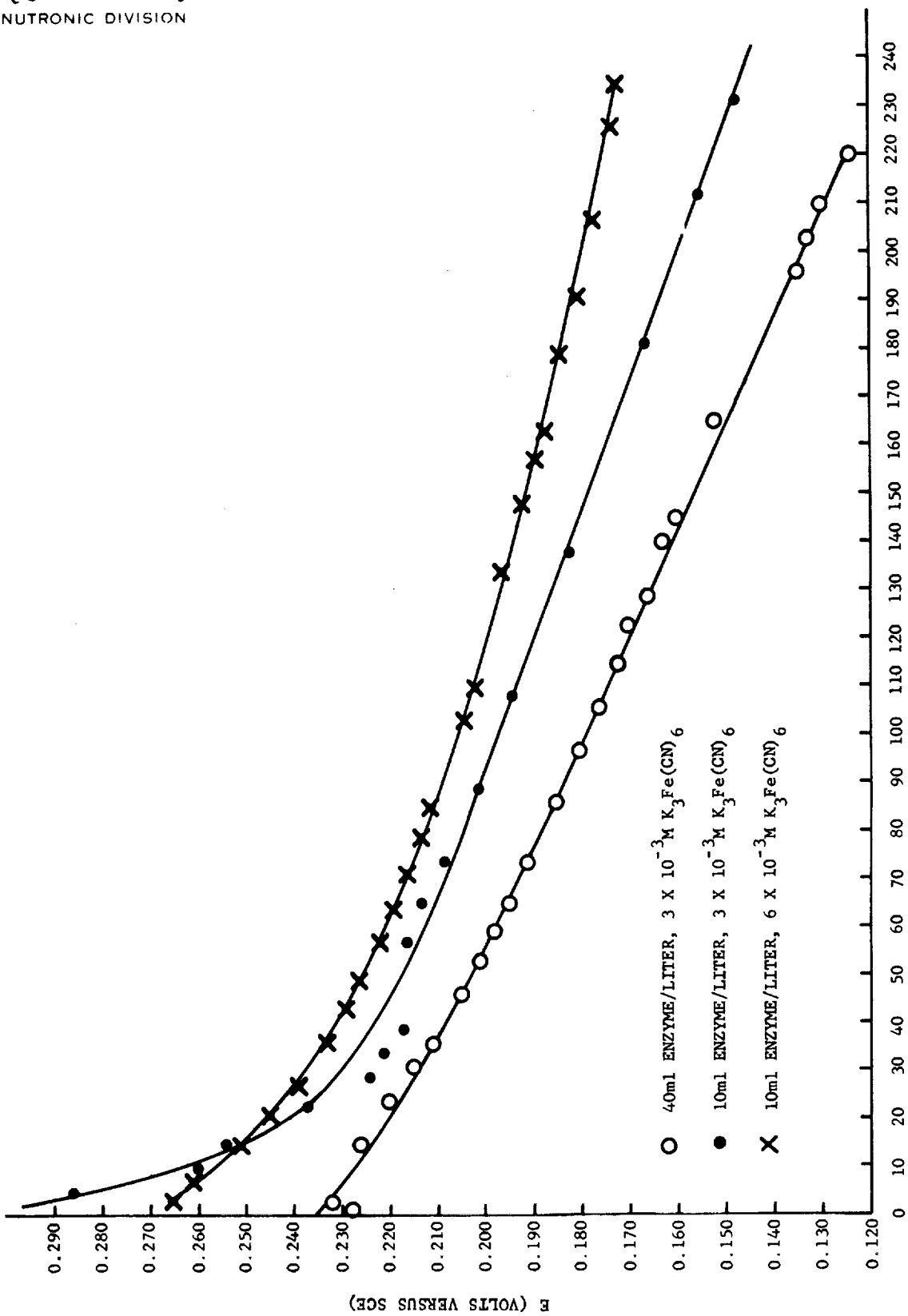


FIGURE 11. OPEN CIRCUIT POTENTIAL CHANGE OF ELECTRODE IN D-AMINO ACID OXIDASE-ALANINE - $K_3Fe(CN)_6$ SYSTEM

curves 2 and 3 initially show similar rates of decrease of E. After about 200 minutes, however, curve 3 shows a substantially decreased rate compared to curve 2. This behavior is in accord with that expected, as discussed above, in the case where the reaction is zero order in ferricyanide. Curves 1 and 2 show a slope which is essentially independent of the enzyme concentration at all times, behavior which is shown by the above treatment to be expected if the reaction is also zero order in the enzyme concentration. The displacement between curves 1 and 2 is probably not of kinetic origin.

Curve 1 was continued beyond the time shown on Figure 11. At 380 minutes, the voltage had reached a value of -0.030 volts, and two additional experiments were carried out at this point.

1. The electrode potential was made positive (vs SCE) by imposing an anodic current which was increased stepwise until a potential of +0.950 was reached. At this point the current was interrupted and the rate of change of voltage observed. It was found to decrease over a period of ten minutes to +0.080v.
2. The cell was repolarized to 0.200v and a current of 340 $\mu$amp drawn for 15 minutes. Current was then interrupted and the decrease in voltage observed again. It was found to decay again to +0.060 (vs SCE) over a period of 17 minutes.

The fast return of the electrode potential from the applied voltages to approximately its original value upon interruption of current indicates that the ferricyanide electrode is appreciably polarized under loads comparable to those which are encountered in these experiments. This fact is of importance in the interpretation of current-voltage data obtained with ferricyanide-enzyme-substrate electrodes. (See Figure 12.)

It must be pointed out that considerable caution must be exercised as regards the significance to be attached to the foregoing kinetic data. This is because of the limited amount of experimental work and the fact that only one run was carried out for each condition so that

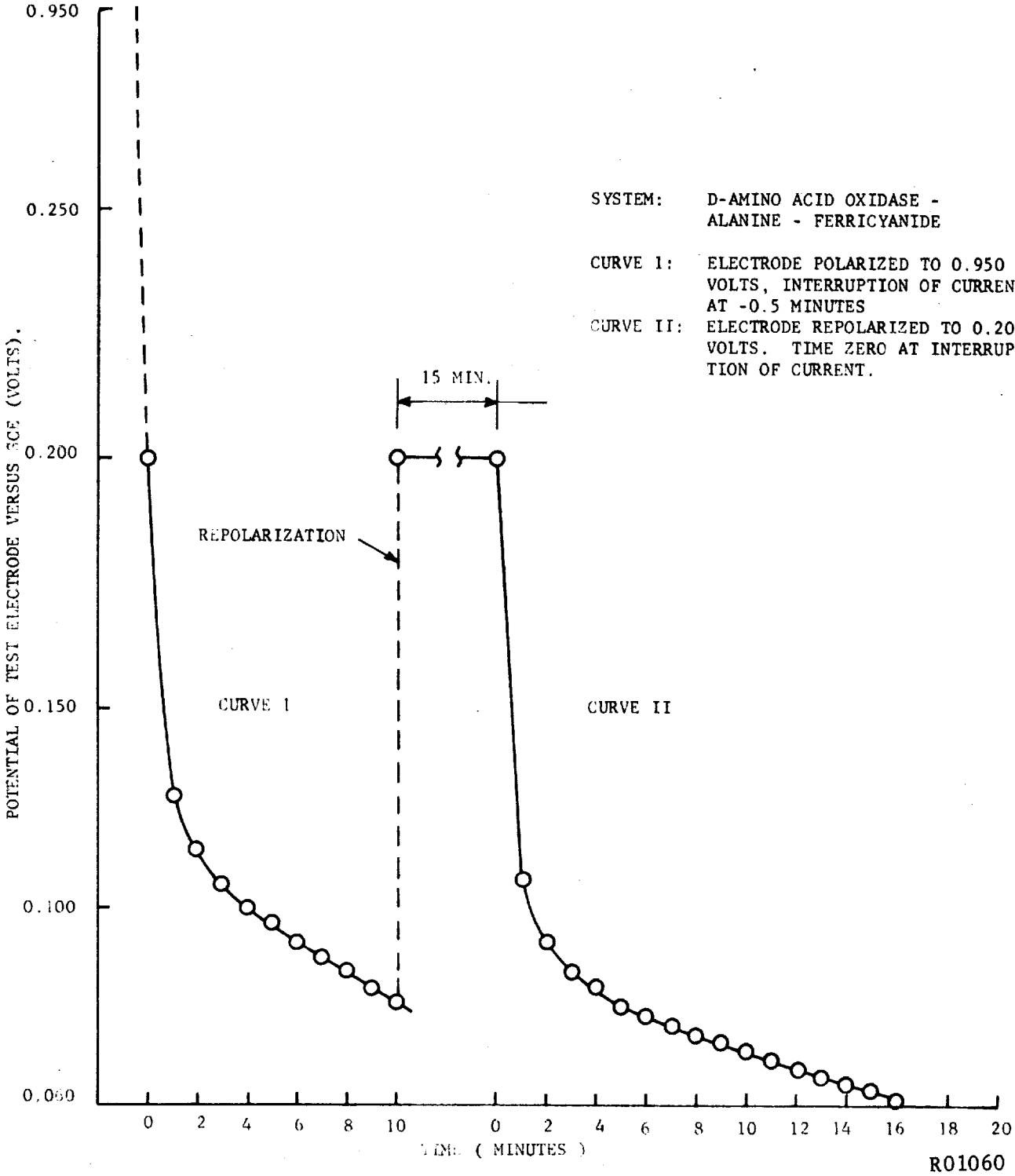


FIGURE 12. CHANGE OF OPEN CIRCUIT POTENTIAL OF TEST ELECTRODE VERSUS SCE AS FUNCTION OF TIME

reproducibility is poorly known. No further work is planned on this problem for the present, since much greater interest and importance attaches to direct bio-electrode reactions.

## 3.5 CHRONOPOTENTIOMETRIC STUDIES

As stated in the fourth monthly progress report the performance of the chronopotentiometric apparatus has been evaluated by the use of a well characterized system, namely, the oxidation of ferrocyanide ion. The transition time, $\tau$, for this system was determined by the method suggested by Delahay.[5] The constancy of $i\tau^{\frac{1}{2}}$ was tested over a wide range of currents for two types of smooth platinum electrodes using an unstirred solution containing $5 \times 10^{-3}$M $K_4Fe(CN)_6$ and $1 \times 10^{-1}$M $KNO_3$. The average deviations of the $i\tau^{\frac{1}{2}}$ values were 4% for the cylindrical platinum electrode and 3.5% for the circular disc platinum electrode with a glass mantel. Reilley and coworkers[2] have reported an average deviation of 1.55% for this system, a value considerably smaller than those obtained in our present experiments. This difference in behavior is not understood at the present time.

# SECTION 4

## DISCUSSION AND CONCLUSIONS

The work carried out to date has been primarily for the purpose of determining what biological systems would be the most suitable subjects for a basic study of bio-electrochemistry. Greatest attention has been directed to amino acid oxidase, but other systems have been examined as well. The data are too incomplete for selection of a suitable system or systems at this time. However, in the course of the work some pertinent observations have emerged.

First, all of the biological oxidation-reduction systems examined were able to produce current in the electrochemical cell under the proper conditions, which in some cases included the presence of an electron transfer agent such as ferricyanide.

Second, in all cases, performance was improved, sometimes to a very large extent, by the presence of an electron transfer agent such as ferricyanide or methylene blue. The inference is that biological substances do not readily undergo oxidation-reduction at electrodes. An explanation of this would constitute an important contribution to the general understanding of bio-electrochemical behavior.

Third, in no case except that of the commercial preparation of amino acid oxidase (Figure 10) was any evidence found of an induction period in the generation of bioelectricity. The inference is that the

source of electrochemical activity is not a long lived intermediate, but must be either a short lived intermediate which rapidly achieves its steady state concentration, or the enzyme system itself.

Fourth, there appears to be only partial correlation between the respiratory activity of an enzyme preparation with different substrates and its electrochemical activity at an electrode. This fact suggests that the reaction mechanisms differ for the two processes as would, indeed, be expected.

These points will be examined further during the course of the program. The limited amount of data presently available does not warrant further attempts at analysis at this time.

# SECTION 5

# PLANS FOR THE NEXT QUARTER

## 5.1 D-AMINO ACID OXIDASE (DAO) STUDIES

The major mechanism of electrical energy production by DAO requires further study. Effort should be made to determine:

1. Whether indole pyruvic acid or parahydroxyphenyl pyruvic acid can act as carriers for reduced flavoprotein, particularly in reactions not normally involving tryptophan (would be of importance, potentially, in a mixed system or whole micro-organism to establish that electron carriers may be formed which are not normally considered).
2. Kinetics of DAO reaction in conjunction with spectrophotometric studies to aid in establishing whether enzyme is involved directly in electrode reaction.
3. Energy output vs. reaction balance to determine cell efficiency and allow conclusions as to the importance of various possible reactions.
4. Differences between electrochemical reaction and normal reaction through tracer studies.

## 5.2 UREASE-UREA SYSTEM

Initial measurements have shown an electrical output with urea-urease which, although low, is still higher than the output obtainable from oxidation of the normal reaction products, i.e. $NH_3$ and $CO_2$, in the electrochemical cell, either in the presence or absence of enzyme. Therefore, it would appear to be a potential case of direct enzyme participation or reaction of an intermediate or unexpected by-product such as carbamide, hydroxylamine, or formamide. Pursuit of this problem should involve

1. Concentration studies of enzyme, substrate, and potential products in relation to electrical output and duration.
2. Effect of inhibitors of urease action.
3. Effect of interposition of semipermeable membrane between enzyme and electrode to prevent direct access of enzyme while permitting product access to electrode.
4. Analogous reactions with amidases, esterases, transamidases, transaminase and other hydrolytic systems.

## 5.3 SCREENING OF OTHER ENZYME OR MULTIENZYME SYSTEMS

Work done so far is not conclusive as to the major mode of action by which electrical energy is produced. However, the wide variety of reactive functions in enzymes makes it desirable to make further surveys of both oxidative and nonoxidative enzymes to determine whether certain enzymes may be capable of direct electrode reaction while others are not. In the survey of nonoxidizing enzymes, those with oxidizable groups, either functional or nonfunctional in normal activity, should be given preference.

## 5.4 WHOLE ORGANISMS

It appears that the initial accent of work with whole organisms should be directed toward determining whether close proximity or attachment of the organism to the electrode has any significant advantage other than that which might be expected from diffusional considerations. As a first approximation in this direction, the studies below may be of value.

1. Effect of localized concentrations of the micro-organism upon electrical output. Comparisons of high concentrations of bacteria, held close to electrode, vs. equivalent amounts distributed in lower concentrations in larger volumes surrounding the electrode.
2. Effects of diffusional barrier systems. Comparisons of activities found with whole cells and carefully prepared whole cell homogenates. Effects of cell wall layers or artificial membranes on electrode upon activities of simple enzymes, multienzyme systems and intact organisms.

## 5.5 ELECTROCHEMICAL METHODS

Work will continue on the chronopotentiometric studies and effort will be direct to improvement in the reproducibility of the $i\tau^{\frac{1}{2}}$ values of the ferrocyanide system. After this has been accomplished the technique will be applied to the study of those biological systems which the survey has indicated to be of greatest interest in terms of bio-electrochemical activity.

## REFERENCES

1. F. L. Crane, Plant Physiology, 32:6, 619-625, 1957.
2. V. Massey, G. Palmer, and R. Bennet, Biochem. Biophys. Acta, 48:1, 1961.
3. R. E. Kay, D. C. Harris, and C. Entenman, U. S. Naval Radiological Defense Laboratory Research and Development Technical Report USNRDL TR-40 NM 006-015.
4. K. Hanabusa, Nature, 189, 551, 1961.
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193:265, 1951.
6. P. Delahay and G. Mamantov, Anal. Chem., 27 478 (1955).
7. C. N. Reilley, G. W. Everett and R. H. Johns, Anal. Chem., 27 483 (1955).
8. A. C. Testa and W. H. Reinmuth, J. Am. Chem. Soc., 83 784 (1961).
9. A. C. Testa and W. H. Reinmuth, Anal. Chem., 32 1518 (1960).
10. P. Delahay, New Instrumental Methods in Electrochemistry, Interscience Publishers, Inc., New York, 1954 pp. 179-216.
11. W. H. Reinmuth, Anal. Chem., 32 1514 (1960).
12. W. H. Reinmuth, Anal. Chem., 33 322 (1961).

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